Smad3 in the Mammary Epithelium Has a Nonredundant Role in the Induction of Apoptosis, but not in the Regulation of Proliferation or Differentiation by Transforming Growth Factor-β

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Abstract

Transforming growth factor-β (TGF-β) regulates proliferation, morphogenesis, and functional differentiation in the mammary gland and plays complex roles in mammary tumorigenesis. Here we show that the signaling mediators Smad1–Smad5 are expressed at all stages of mammary gland development. To begin to investigate which Smads mediate which TGF-β responses, we have analyzed mammary gland development in Smad3 null mice. Smad3 null virgin females showed delayed mammary gland development. However, this phenotype was secondary to ovarian insufficiency because Smad3 null mammary epithelium developed normally in hormonally supplemented Smad3 null mice or when transplanted into wild-type hosts. Absence of Smad3 had no effect on the ability of TGF-β to inhibit the growth of mammary epithelial cells in culture, and no compensatory changes in expression or activation of Smad2 were seen in the Smad3 null epithelium. A small but significant decrease in apoptotic cells was seen in involuting glands from Smad3 null transplants. The results suggest that epithelial Smad3 is dispensable for TGF-β effects on proliferation and differentiation in the mammary gland, but that it contributes in a nonredundant manner to the induction of apoptosis.

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Introduction

TGF-β[3] is a member of a superfamily of highly pleiotropic proteins that regulate growth, development, and functional differentiation in multiple organ systems (1). One key property of TGF-β is the ability to inhibit the proliferation of epithelial cells, and considerable evidence has emerged suggesting that the TGF-β ligand/response system constitutes a novel tumor suppressor pathway (2, 3). This role is particularly clear for tissues of the gastrointestinal tract, but the TGF-β system is also implicated in less penetrant or more complex ways in tumorigenesis in many other organ systems, including the breast.

In the mouse mammary gland, the three mammalian isoforms of TGF-β are expressed at all stages of development except lactation (4), and they play multiple roles in glandular development and homeostasis. These include regulating stem cell kinetics and the establishment of proper mammary gland architecture, preventing inappropriate functional differentiation in the virgin mouse and inducing apoptosis in the involuting gland (5–9). There is direct experimental evidence that TGF-β has tumor suppressor activity in the mouse mammary gland. Transgenic overexpression of TGF-β1 suppresses tumorigenesis, whereas reducing epithelial responsiveness to TGF-β enhances the process (10, 11). Correlative data suggest this is also true in the human breast. Decreased expression of TβRII in early-stage proliferative lesions correlates with increased risk of subsequently developing invasive breast cancer, whereas loss of TβRII in ductal carcinoma *in situ* and invasive breast cancer correlates with more aggressive disease (12, 13). However, TGF-β has also been implicated as a pro-oncogenic agent in late-stage cancers in many organs, which suggests a complex dual role during tumorigenesis (2). Advanced human breast cancers show increased expression of TGF-β1, and TGF-β can promote the metastasis of certain breast cancer cell lines (14–17). The molecular mechanisms underlying the switch in TGF-β function from tumor suppressor to oncogene in the later stages of tumorigenesis are not known.

The TGF-β superfamily members signal through transmembrane serine-threonine receptor kinases, which in turn activate multiple downstream signal transduction pathways (reviewed in Refs. 2, 3, 18). The predominant pathway involves a family of signal transduction components termed Smads. The activated type I receptor kinases bind to and phosphorylate members of a family of receptor-restricted Smads (Smads1, -2, -3, -5, and -8), which then interact with

[3] The abbreviations used are: TGF-β, transforming growth factor β; TβRII, TGF-β type II receptor; MEC, mammary epithelial cell.

a common mediator Smad (Smad4) and translocate to the nucleus, in which they induce changes in transcription of target genes. Smads2 and 3 primarily mediate responses to TGF-β, activins and nodal, whereas Smads1, -5, and -8 normally mediate responses to the bone morphogenetic family of proteins (18). However, Smad1 has also been implicated downstream of TGF-β in human breast cancer cells (19).

Recent studies have suggested that different Smads may be required for different biological responses to TGF-β (20). Interestingly, it also appears that the specific Smad that is used for a given response may be cell-type dependent. For example, the inhibition of T-cell proliferation by TGF-β is absolutely dependent on the presence of Smad3, whereas the inhibition of B-cell proliferation is not (21). To understand which Smad proteins are necessary for the various functions of TGF-β in mammary tissue, we have begun a systematic analysis of mammary phenotypes and tumorigenesis in the various Smad null mice. Ultimately, we hope this approach will give insight into whether the tumor suppressor and oncogenic activities of TGF-β in the mammary gland use different signal transduction pathways. Although homozygous deletion of Smads1, -2, -4, and -5 causes early embryonic lethality, Smad3 null mice survive to adulthood (22). Here, we use these mice to demonstrate that TGF-β can regulate proliferation, development, and functional differentiation in the mammary gland in the absence of epithelial Smad3, but that Smad3 contributes in a nonredundant way to the induction of apoptosis during postlactational involution.

Results

Smads1–5 Are Expressed in the Mammary Gland at All Stages of Development. Northern blot analysis of Smad expression in mammary tissue shows that Smads1–5 are expressed at all stages of development (Fig. 1). Expression of all five Smads is decreased in the lactating gland, and for Smads2, -4, and -5, this decreased expression persists through involution day 2 but is restored again by involution day 6. Apart from this, expression is relatively constant throughout development. All five Smads are also present in the cleared mammary fat pad. Unlike the other Smads, Smad3 mRNA shows a substantial enrichment in the intact mammary tissue when compared with the cleared fat pad. This suggests that Smad3 may be particularly highly expressed in the mammary epithelium. The absence of cytokeratin 18 mRNA in the fat pad sample confirms that these were effectively cleared of epithelium.

Smad3 Null Mice Have Underdeveloped Mammary Glands. A significant fraction (~70%) of Smad3 null mice are runted at weaning, develop a wasting syndrome associated with impaired mucosal immunity and abscess formation, and die at between 1 and 3 months of age (21). These mice were not used for the analysis. However, the remaining Smad3 nulls appear to develop normally, although slightly smaller than their littermates, and live for 3–8 months before ultimately succumbing to the same syndrome but with a later onset. The Smad3 null mice used for this analysis were matched for weight as closely as possible with their wild-type controls, although as a group, the Smad3 nulls were on

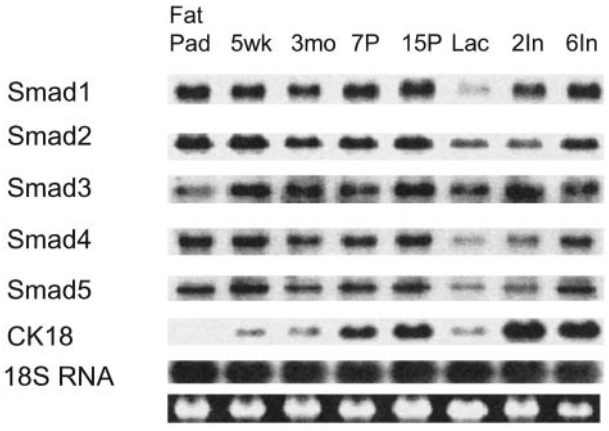

Fig. 1. Smad1–5 mRNAs are expressed in the mammary gland at all stages of development. RNA was prepared from the mammary glands of mice harvested at the following developmental stages: cleared fat pad (*Fat Pad*), early puberty (*5wk*), mature virgin (*3mo*), 7-day pregnant (*7P*), 15-day pregnant (*15P*), 1-day lactating (*Lac*), 2-day involution (*2In*) and 6-day involution (*6In*). Northern blots of total RNA were probed for *Smad1–Smad5*. The cytokeratin 18 (*CK18*) probe was used to determine epithelial content of the sample, and the *18S rRNA* band was used as a loading control.

average about 10% lighter. Whole mount analysis of the mammary glands from these mice showed a phenotype of delayed ductal development and decreased side-branching and alveolar bud formation in virgin animals when compared with age-matched wild-type littermates (Fig. 2). This phenotype was evident in puberty (6.5–7.5-week-old virgins) and persisted into adulthood (4–6.5-month-old virgins). It is the opposite of what would be predicted for a TGF-β functional null but shows some similarities to the activin βB null mammary phenotype (23). This raised the possibility that activin might signal predominantly through a Smad3-dependent pathway in the mammary gland, whereas TGF-βs might use other pathways. Alternatively, the unexpected mammary phenotype could be secondary to other changes in the Smad3 null mice.

Ovarian Function Is Abnormal in Smad3 Null Mice. Smad3 null mice showed reduced fertility, and successful pregnancies were rare. Because estrogen is required for proper mammary ductal development (24), serum levels of 17-β-estradiol were determined for 8–10-week-old female mice of both genotypes in estrus. Whereas wild-type mice had 63.8 $\pm$ 18.9 pg/ml of 17-β-estradiol in their serum (n = 4), the Smad3 null mice had only 30.8 $\pm$ 4.7 pg/ml (n = 5; P = 0.007, t test for independent samples). This observation suggests that the Smad3 null mice have impaired ovarian function. A detailed analysis of the ovaries showed that the Smad3 null mice have fewer antral follicles than do wild-type mice, which suggests that the absence of Smad3 delays follicular maturation (25).

Smad3 Is Not Necessary for Normal Mammary Gland Development in a Hormonally Replete Environment. To determine whether the mammary phenotype of the Smad3 null mice was secondary to the ovarian insufficiency, two approaches were taken. In the first, Smad3 null mammary gland fragments were transplanted into the cleared fat pad of

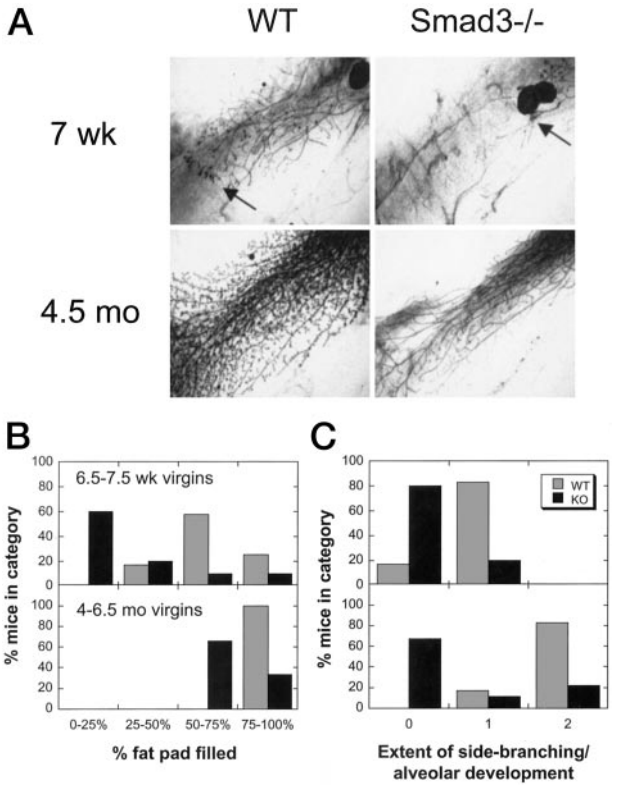

Fig. 2. Smad3 null mice have underdeveloped mammary glands. *A*, whole-mount analysis of wild-type and Smad3 null mice during puberty (7 week) or in adult virgins (4.5-month). *Arrows*, the growing end of the ductal tree in pubertal mice. In Smad3 null mice at 7 weeks, the ductal tree has not penetrated beyond the lymph node. *B*, effect of *Smad3* genotype on the extent of penetration of the fat pad by the ductal tree in pubertal (6.5–7.5-week) or adult (4–6.5-month) virgin female mice. *C*, effect of *Smad3* genotype on the degree of ductal side-branching and alveolar bud formation in pubertal or adult virgin female mice. *Category 0*, little or no side-branching or budding; *category 1*, moderate; *category 2*, extensive. ▨, wild-type (*WT*) mice; ■, Smad3 null (*KO*) mice. For the 6.5–7.5-week time point, $n = 12$ for WT mice and $n = 10$ for Smad3 nulls; at the 4–6.5-month time point, $n = 6$ for WT and $n = 9$ for Smad3 nulls.

wild-type hosts. Smad3 null epithelium developed normally when transplanted into wild-type hosts and was essentially indistinguishable from the wild-type control transplants. This was true for transplanted glands allowed to develop for 4 weeks (Fig. 3, *A* and *B*) or 8 weeks in virgin hosts (not shown), and for transplanted glands at day 7 (Fig. 3, *C* and *D*) and day 14 of pregnancy (not shown), and day 1 of lactation (Fig. 3, *E* and *F*). This suggests that epithelial Smad3 is not necessary for normal mammary gland development and functional differentiation. Previous experiments have suggested that the inhibitory effects of TGF-β on ductal morphogenesis are mediated *via* effects on the stroma (7). To address the possible importance of stromal Smad3 in ductal morphogenesis, slow-release pellets containing estrogen and progesterone were transplanted into 4.5-week-old Smad3 null and wild-type females, and mammary glands were harvested for whole-mount analysis 3 weeks later. No significant differences were observed between the morphology of Smad3 null and wild-type glands after hormone supplementation for mice that were of comparable weight at the end of the experiment ($n = 3$ for each genotype; data not shown).

Smad3 Is Not Necessary for the Growth-inhibitory Effects of TGF-β on MECs. TGF-β is a potent inhibitor of the growth of many cell types, including MECs (26). We found that basal proliferation in primary MECs derived from Smad3 null mice was consistently 20–30% lower than those from wild-type mice (data not shown). We assume that this reflects the slightly smaller size and poorer health status of the Smad3 null mice. However, MECs derived from Smad3 null mice were growth inhibited by TGF-β to the same extent as wild-type MECs, which was typically 30–45% depending on the experiment (Fig. 4). Activin A had no effect on the proliferation of either wild-type or Smad3 null MECs (data not shown).

There Are No Major Compensatory Changes in Expression or Activation of Smad2 in the Mammary Gland of the Smad3 Null Mouse. Smad2 and Smad3 are structurally very closely related and have both been implicated in signaling from TGF-β. To investigate the possibility that increased expression or activation of Smad2 might compensate for a loss of Smad3, Smad mRNA expression was compared in mammary glands from adult virgin Smad3 null and wild-type mice. No changes in expression of Smads1, -2, -4, or -5 were seen at the mRNA level (Fig. 5*A*). Western blot analysis of phospho-Smad2 levels in primary MECs after the addition of TGF-β also showed no compensatory increase in the level of phosphorylated, activated Smad2 in Smad3 null MECs when compared with wild-type MECs.

Smad3 Contributes to the Induction of Apoptosis during Involution. TGF-β3 is induced early in involution and plays a role in the induction of apoptosis in the involuting gland (9). To determine whether Smad3 is necessary for this role of TGF-β, Smad3 null mammary epithelium was transplanted into the cleared no. 4 mammary fatpads of nude mouse hosts, and wild-type epithelium was transplanted into the cleared no. 9 fatpad on the contralateral side. Mice were cycled through pregnancy. Because the transplants lack a nipple, milk accumulates in the transplanted gland at parturition, and involution is rapidly induced because of milk stasis. Apoptosis in the involuting glands was quantitated in transplants on days 1 and 2 after parturition. The percentage of apoptotic cells in the involuting transplants varied considerably between mice ($3.7 \pm 3.0\%$ for Smad3 null glands *versus* $5.1 \pm 3.2\%$ for wild-type glands). However, for the matched pairs of Smad3 null and wild-type transplants in any given mouse, the wild-type glands consistently had significantly more apoptotic cells than did the Smad3 null glands (wild-type/Smad3 null $= 1.71 \pm 0.80$; $n = 13$; $P = 0.04$, the Sign test).

Discussion

Smad3 Is Not Required for the Normal Development and Functional Differentiation of the Mammary Gland. In the present study, we have shown that Smads1–5 are expressed in the mammary gland at all stages of mammary development, and that Smad3 appears to be particularly highly expressed in the mammary epithelium. Mammary glands in the Smad3 null mice are underdeveloped. However, we have

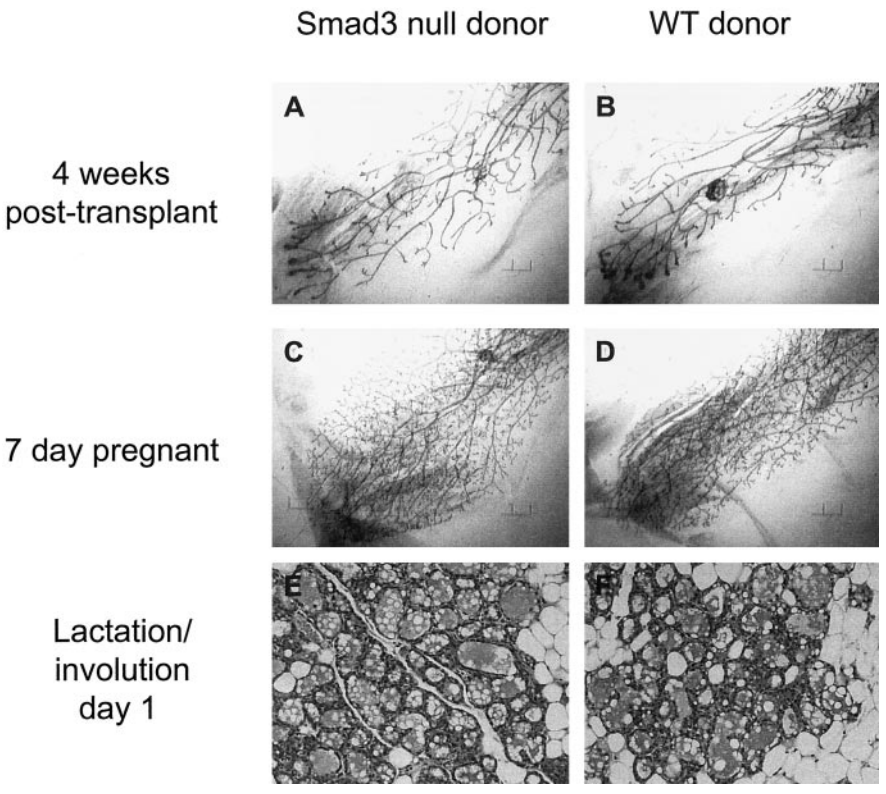

Fig. 3. Smad3 null mammary epithelium develops normally in wild-type hosts. Smad3 null (*A*, *C*, and *E*) or wild-type (*B*, *D*, and *F*) mammary fragments were transplanted into the cleared fat pad of wild-type hosts and were allowed to develop for the specified time before transplanted glands were harvested for analysis. *A–D*, whole mounts of transplanted glands at 4 weeks posttransplantation (*A*, *B*), or day 7 of pregnancy (*C*, *D*). *E*, *F*, H&E-stained sections of lactating transplanted glands harvested 1 day after parturition (×20).

shown that this phenotype is secondary to ovarian insufficiency, because the Smad3 null mammary epithelium is capable of developing normally and undergoing full functional differentiation when given appropriate hormonal stimulation. Previous work had suggested that TGF-β acts directly on the virgin mammary epithelium to prevent inappropriate lactational differentiation, and acts indirectly via the stroma to limit ductal branching (6, 7). We have shown that neither of these effects are Smad3-dependent, because Smad3 null epithelium transplanted into wild-type hosts did not undergo precocious lobulo-alveolar development, and hormonal supplementation of intact Smad3 null mice resulted in normal ductal development without increased branching. Smad3 is also used for signaling by the activins/inhibins and by the morphogen nodal in addition to the TGF-βs (27). Currently, it is not known whether the nodal is expressed in the mammary gland, or whether it might play any role there. However, stromally derived activin/inhibin βB is required for ductal elongation and alveolar morphogenesis (23). Our results further indicate that epithelial Smad3 is not required for any stimulatory effects of activin βB on ductal development or functional differentiation.

The Role of Smad3 in Growth Inhibition and Apoptosis of the Mammary Epithelium. The ability of TGF-β to inhibit the proliferation of epithelial cells is important for its tumor suppressor effects. Suppression of mammary tumorigenesis in mice overexpressing TGF-β1 in the mammary gland is associated with a 10-fold reduction in the labeling index in the epithelium of mature virgins (10), and in clinical breast cancer specimens, there is an inverse correlation between TβRII expression and mitotic count (12). In other tissues, the relative importance of Smad3 in growth inhibition by TGF-β varies among cell types. For example, inhibition of proliferation by TGF-β is absolutely dependent on Smad3 in T cells, partially dependent in keratinocytes, and completely Smad3-independent in B cells (21, 28). Our data show that Smad3 is not required for the growth-inhibitory effects of TGF-β on MECs.

TGF-β can induce apoptosis in many cell types, and this property is likely also to contribute to tumor suppressor mechanisms (reviewed in Ref. 2). We have been unable to find conditions under which TGF-β induces apoptosis, as opposed to growth inhibition, in primary MECs in culture. However, it is known that endogenous TGF-β3 induces apoptosis in the early stages of mammary gland involution (9). Here we have shown that the absence of Smad3 causes a statistically significant 30% reduction in the number of apoptotic epithelial cells during days 1 and 2 of involution in transplanted mammary glands. A 2-fold greater reduction in apoptosis was observed in glands lacking TGF-β3 (9). Together, the data suggest that Smad3 contributes to, but is not absolutely required for, the induction of apoptosis by TGF-β in the involuting mammary gland. Smad3 has also been implicated in the induction of apoptosis by TGF-β in hepatoma and myeloma cell lines (29).

Redundancy among Smads and Involvement of Other Pathways. The mild mammary phenotype of the Smad3 null mouse is somewhat unexpected given the relatively high levels of expression of Smad3 in the mammary epithelium. These observations could be rationalized if there were func-

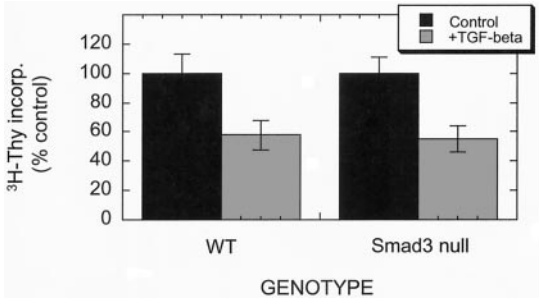

Fig. 4. Smad3 is not necessary for growth-inhibitory effects of TGF-β on the mammary epithelium. Primary MECs derived from Smad3 null or wild-type mammary glands were grown in the presence and absence of 5 ng/ml TGF-β1. Proliferation was determined by the incorporation of [^{3}H]thymidine into DNA, and was normalized to the no-TGF-β condition in each case. Data are the mean $\pm$ SD of three determinations and are representative of two replicate experiments.

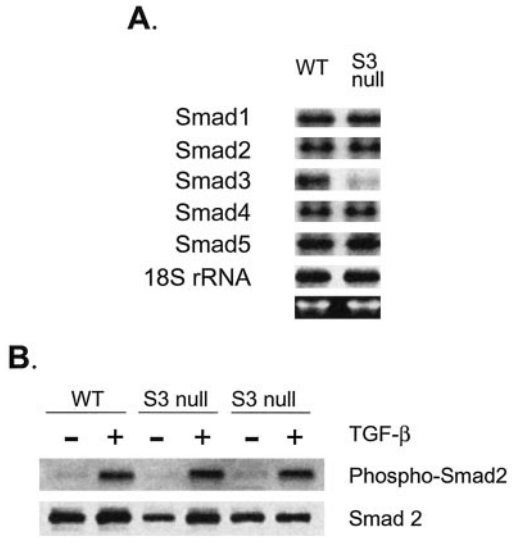

Fig. 5. Smad3 null mammary tissue show no compensatory changes in expression or activation of other Smads. *A*, Northern blot analysis of Smad mRNA expression in the mammary glands of adult virgin wild-type (*WT*) and Smad3 null (*S3 null*) mice. *B*, Western blot analysis of Smad2 activation in primary MECs derived from wild-type (*WT*) or Smad3 null (*S3 null*) mice after stimulation with 5 ng/ml TGF-β1 for 30 min. Data are shown for two independent isolates of Smad3 null cells.

tional redundancy among the receptor-activated Smads, or between the Smad pathway and alternative signal transduction pathways. Indeed, if TGF-β were critical for the maintenance of proliferative homeostasis in the mammary gland, some degree of redundancy in the mechanisms for propagation of the TGF-β signal to the nucleus could provide an important safeguard against loss of tumor suppressor function. Smad2 and Smad3, the two Smads activated by the type I TGF-β receptor, are structurally very similar, with 91% identity in amino acid sequence. However the phenotypes of the Smad2 and Smad3 null mice are quite distinct (22), and different genes are regulated by different Smads or combinations of Smads *in vitro* (20). For example, in mouse embryo fibroblasts, induction of *matrix metalloproteinase-2* by TGF-β was selectively dependent on Smad2, whereas induction of c-*fos* was dependent on Smad3, and both Smad2 and Smad3 were required for induction of *plasminogen activator inhibitor-1* (20). These data suggest that, in general, the two Smads are not functionally interchangeable, as is also suggested by the finding that the Smad2/Smad3 compound null heterozygote is embryonic lethal, whereas the Smad3 null homozygote is not (30). However, the issue of whether Smad2 and Smad3 show any functional redundancy in the mammary gland can be definitively addressed only when the Smad2 conditional null and Smad2/3 conditional double null mice become available. These mice will also allow any unique roles for Smad2 in the mammary gland to be determined.

In contrast to the lack of general evidence for functional redundancy between Smad2 and Smad3, there are precedents for redundancy between Smad-mediated and non-Smad pathways in transducing TGF-β signals *in vitro*. For example, TGF-β can inhibit the proliferation of some epithelial cells through both a Smad-dependent pathway and the protein phosphatase 2A/p70s6 kinase pathway, and both must be inactivated for the growth-inhibitory effect to be lost (31). Additional studies with pathway-specific inhibitors will help determine whether other signal transduction pathways can substitute for the Smad3 path in the regulation by TGF-β of proliferation and differentiation in the mammary gland, or whether Smad3 is simply not involved in these functions. However, our data clearly show that Smad3 makes a nonredundant contribution to the ability of TGF-β to induce apoptosis in the involuting mammary gland. Comparing our results with previous work (9), we estimate that Smad3 is absolutely required for ~50% of the apoptotic response to endogenous TGF-β during early-stage involution.

Possible Implications for Tumorigenesis. In contrast to Smad2, which is inactivated in a significant fraction of colon and lung tumors, and Smad4, which is inactivated or deleted in nearly one-half of all pancreatic tumors, Smad3 has not been found to be mutated or deleted in any human solid tumors (2, 3). This suggests either that Smad3 may not be important for tumor suppression in most epithelial tumors, or that its loss may additionally compromise other functions that are required for efficient tumor progression. Smad3 mice with a targeted disruption of exon 2 have been reported to develop metastatic colorectal cancer with high penetrance at between 4 and 6 months of age (32), but the other two independently derived Smad3 null mouse lines do not seem to be cancer prone (21, 33). We have not seen any spontaneous mammary tumorigenesis in our Smad3 null colony, although the mice do not live beyond 8 months of age, and they are extremely difficult to breed, so we have been able to make our observations only on relatively young virgin mice. However, combined with the absence of Smad3 mutations in human breast cancer and the lack of requirement for Smad3 for growth inhibition, our data suggest that Smad3 is probably not a critical mediator of the tumor suppressor function of TGF-β in the mammary gland. With longer-lived conditional Smad3 nulls, it will be interesting to determine whether Smad3 is necessary for any of the pro-oncogenic effects of TGF-β in late-stage mammary cancer. In relation to this

question, the induction of parathyroid hormone-related peptide by TGF-β in MDA-MB-231 breast cancer cells has been shown to be dependent on a synergism between Smad3 and Ets-1, which suggests that the ability of TGF-β to promote bony metastases may require Smad3 (34). If tumor suppressor and oncogenic activities of TGF-β in the mammary gland use different Smads, it may be possible to manipulate these pathways independently in novel preventive or therapeutic approaches to the problem of breast cancer. The development of Smad conditional null mice will provide invaluable tools for addressing these questions.

Materials and Methods

Smad3 Null Mice. The generation of Smad3^{delEx8} null mice by homologous recombination was described previously (21). In this line of mice, exon 8 of the *Smad3* gene is deleted. The deletion removes the L3 loop, which is necessary for interaction with the TGF-β receptors, and the COOH-terminal SSVS consensus phosphorylation site. Hemizygous mutant mice were mated to generate null mice. All of the mice analyzed were on a mixed C57Bl/6 $\times$ 129Sv background, except for some mice used as transplant donors, which were pure 129Sv. For timed pregnancies, mice were mated and inspected daily for vaginal plugs. The day on which plugs were observed was counted as day 0 of pregnancy.

Whole Mounts and Histology. The first inguinal gland (no. 4) was removed at the indicated times of development and spread on a glass slide. After fixation for 2–4 h in Carnoy's solution, glands were hydrated and stained with Carmine alum and dehydrated and mounted as described previously (8). Whole mounts were directly imaged with a charge-coupled device camera mounted on a Zeiss ICM405 microscope. The fraction of the fat pad filled by epithelium in developing glands was determined by visual inspection, with the lymph node as a reference point. Similarly the degree of side-branching and/or alveolar budding was determined to fall into one of three categories: 0 (none), 1 (moderate), or 2 (extensive), by comparison with three previously selected whole mounts. The contralateral inguinal gland (no. 9) was fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and sectioned for histology or immunohistochemistry.

RNA Isolation and Northern Blots. Total RNA was isolated from mammary glands of different developmental stages using the TRIzol reagent according to the manufacturer's instructions (Life Technologies, Inc. Inc.). The no. 4 and no. 9 glands were harvested from 3–5 mice/group. Total RNA (10 μg/lane) was separated on 1.0% agarose/formaldehyde gels and blotted onto Nytran membranes. The blot was hybridized with [^{32}P]dATP-labeled cDNA probes, which were generated using the Ambion StripAble DNA Probe Synthesis and Removal system according to the manufacturer's instructions. After hybridization, the membrane was exposed to film or PhosphorImager. The membrane was then stripped and reprobed with a different probe. A 1.1-kb fragment of the mouse cytokeratin 18 cDNA (generous gift of Dr. Robert Oshima, The Burnham Institute, La Jolla, CA) was used as a probe to determine the presence of epithelial cells, and an 18S RNA probe was used as a loading control. Murine cDNA probes for Smad1 (bases 650–901), Smad2 (bases 581–881), Smad3 (bases 809-1145), Smad4 (bases 731-1281), and Smad5 (bases 631-1008) were the generous gift of Drs. Gillian Ashcroft and Amy Cao, National Cancer Institute, NIH, Bethesda, MD.

Generation of Cleared Fat Pads and Transplantation. For preparation of RNA, the inguinal fat pads of 3-week-old female mice were cleared of endogenous epithelium as described previously (35), and the epithelial-free portion of the fat pad was harvested. The absence of epithelium was confirmed by the absence of epithelial-specific cytokeratin 18 RNA on Northern blots. For transplantation of mammary epithelium, the inguinal fat pads of 3-week-old wild-type 129Sv female mice were cleared as above. Small ($\sim$1.5 $\times$ 1.5 mm) portions of mammary gland were isolated from 8-week-old 129Sv wild-type or Smad3 null mouse donors and were implanted into the cleared fat pads of the recipient mice. Each recipient mouse had a Smad3 null donor implant on one cleared fat pad and a wild-type implant on the contralateral fat pad. Four weeks (two mice) or 8 weeks (four mice) after transplantation, the transplanted tissue was harvested for whole-mount analysis. Alternatively, 2 months after transplantation, host mice were mated, and transplanted tissue was harvested at day 7 of pregnancy (five mice), day 14 of pregnancy (one mouse), and day 1 of lactation (one mouse) for whole-mount analysis. For studies on involution, Smad null and wild-type donor mice were of mixed C57Bl/6 $\times$ 129Sv background because of the poor availability of pure 129Sv nulls, and donor mammary gland fragments were transplanted to NCR nu/nu immunodeficient hosts as above. Eight weeks after transplantation, host mice were mated and cycled through pregnancy, and glands were harvested on day 1 (seven mice) and day 2 (six mice) after parturition. Transplanted tissue was fixed in neutral buffered formalin for analysis of histology and apoptotic indices. Apoptotic nuclei were detected by *in situ* end-labeling catalyzed by terminal deoxytransferase using the Apoptag *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions and were counterstained with methyl green. The number of apoptotic nuclei in 25 high-powered fields ($\times$40) were manually counted for each transplant. Apoptotic indices were calculated using ImagePro software to estimate the total number of epithelial cells in each field. This was done by defining a color threshold that identified the counterstained epithelial cells, quantitating the epithelial area for each field, and then normalizing to the number of epithelial cells in the field. Normalization was done using a value for the average area occupied by 100 epithelial cells, as determined by counting cells/area for five distinct fields containing 40–200 cells.

Determination of Endogenous Estradiol Levels and Hormone Supplementation. Serum was collected from 8–10-week-old Smad3 null and wild-type mice in estrus. Mice were determined to be in estrus by vaginal lavage. Serum levels of β-estradiol were determined using a 17-β-estradiol immunoassay kit (R&D Systems Inc., Minneapolis, MN). To increase endogenous ovarian hormone levels, 21-day slow-release pellets containing 10 mg of progesterone and 0.01 mg of 17-β-estradiol (Innovative Research of Amer-

ica, Sarasota, FL) were implanted s.c. on the backs of 4.5-week-old mice. Mammary glands were harvested for whole-mount analysis 3 weeks after the implantation of the pellet.

Primary Cultures of MECs and Growth-inhibition Assays. Primary cultures of MECs were prepared from 6–8-week-old female mice, essentially as described previously (8). MECs were suspended to 10^5 cells/ml in growth medium consisting of DMEM, 10% fetal bovine serum, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, and penicillin/streptomycin, and were seeded in 24 well plates. After 24 h, cells were switched to assay medium containing DMEM, 0.2% fetal bovine serum, 5 μg/ml insulin, 10ng/ml epidermal growth factor, 10 mM HEPES (pH 7.4) and penicillin/streptomycin, with or without the addition of 5 ng/ml TGF-β1 (R&D Systems Inc.) or 5 ng/ml recombinant human activin A (National Hormone and Pituitary Program, Torrance, CA). After an additional 22 h., cells were pulsed with [^{3}H]thymidine for 2 h and then harvested as described previously (36), to determine the extent of incorporation of [^{3}H]thymidine into DNA.

Western Blot Analysis. Primary MECs were prepared as above and seeded in 60-mm dishes. Cells were treated with 5 ng/ml TGF-β for 30 min before harvesting the cell layer into ice-cold modified radioimmunoprecipitation assay buffer containing phosphatase inhibitors [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM sodium vanadate, and 1 mM sodium fluoride]. Clarified lysate (30 μg protein/well) was run on 8% Tris-glycine gels under reducing conditions and blotted onto nitrocellulose. Western blots were probed with a rabbit polyclonal antiphospho Smad2 IgG fraction (no. 06-829, Upstate Biotechnology, Lake Placid, NY) or a rabbit polyclonal anti-Smad2 IgG (Zymed Laboratories Inc., So. San Francisco, CA), and developed using the SuperSignal West Pico detection system (Pierce, Rockford IL).

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